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Degradation of Human Immunoglobulins by Proteases from *Streptococcus pneumoniae* Obtained from Various Human Sources

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The ability of *Streptococcus pneumoniae* to degrade human secretory immunoglobulin A (S-IgA), IgG, and IgM was tested in 102 strains by use of the thin-layer enzyme assay cultivation technique. The strains were isolated from patients with acute phases of otitis media, meningitis, and pneumonia as well as from symptomless carriers. An ability to degrade S-IgA, IgG, and IgM was revealed in 50, 84, and 96 strains, respectively. An IgG- and IgM-degrading ability of *S. pneumoniae* has not previously been reported. A concurrent degradation of the three immunoglobulins was revealed in 38 strains; degradation of two of them was revealed in 54 strains, and degradation of only one of them was revealed in 9 strains. One strain failed to degrade any of the immunoglobulins. Correlations were not found between the ability of the *S. pneumoniae* strains to degrade S-IgA, IgG, or IgM and the serotype affiliation or between the ability to degrade IgG or IgM and the origin of strains. However, the ability to degrade S-IgA was evident more often in strains isolated from symptomless carriers and from bronchial secretions of patients with acute pneumonia than it was in strains from patients with acute meningitis or acute otitis media or from the blood of patients with acute pneumonia. These latter findings may indicate a biological significance of S-IgA-degrading ability in bacterial colonization of mucosal surfaces.

Immunoglobulin-degrading proteases constitute a group of microbial enzymes that has gained much interest due to their potential significance as virulence factors (8, 13, 21; M. Kilian, B. Thomsen, T. E. Peterson, and H. S. Bleeg, N.Y. Acad. Sci., in press). Such enzymes have been assumed to facilitate both bacterial colonization on mucous membranes and penetration of bacterial cells and their antigenic products through the mucosal barrier by elimination of immunoglobulins. An increasing amount of data supports these assumptions (8, 13; Kilian et al., in press). Consequently, immunoglobulin-degrading enzymes have been demonstrated mainly in pathogenic species and in species closely associated with infectious diseases (8, 11, 19).

Streptococcus pneumoniae is one of the major pathogens in pneumonia, meningitis, sinusitis, and otitis media and also gives rise to systemic infections (7). This pathogen has been shown in several studies to produce an extracellular human immunoglobulin A1 (IgA1)-specific protease (10, 11, 16). More than 50 strains of *S. pneumoniae* have been examined, and all degraded IgA1. Examination of the secretory IgA (S-IgA)-, IgG-, and IgM-degrading ability of *S. pneumoniae* has, on the contrary, been performed in only a few strains.

The aim of the present study was to test the ability of *S. pneumoniae* to degrade human S-IgA, IgG, and IgM. The strains used were isolated from samples taken from different types of human infections as well as from healthy human carriers and were of various serotypes.

MATERIALS AND METHODS

Bacteria. One hundred and two strains of *S. pneumoniae* isolated from patients in Sweden were included in the study (Table 1). The bacterial species were identified by the method of Cowan and Steel (2). Thirty-eight strains were isolated from nasopharyngeal swabs of patients with acute otitis media. Eighteen strains were from the cerebrospinal fluid of patients with acute bacterial meningitis. Twenty-eight strains were from blood cultures, and nine strains were from bronchial secretions of patients with radiographically verified acute pneumonia. Ten strains were from throat swabs of individuals without signs of infection.

After the primary isolation on blood agar medium, the bacteria were lyophilized and stored. This was kindly performed by E. Falsen (Department of Clinical Bacteriology, University of Göteborg, Göteborg, Sweden). Counterimmunoelectrophoresis and the capsular reaction test (6, 15) were used for serotyping. Confirmations of the typing and, for some strains, final typing were kindly performed by J. Henrichsen (Statens Seruminstitut, Copenhagen, Denmark).

The lyophilized bacteria were subcultivated twice on horse blood agar plates before they were tested for production of immunoglobulin-degrading enzymes.

Immunoglobulins. S-IgA was prepared from human breast milk (500 ml) collected from two healthy mothers. The pH of the milk was adjusted to 4.5 with 1 M acetic acid and then centrifuged at 10,000 × *g* for 1 h. The whey was collected and dialyzed against 0.02 M phosphate buffer at pH 6.8 Ion-exchange chromatography was performed on a DEAE-Sephacel column equilibrated with 0.02 M phosphate buffer at pH 6.8. Elution was performed stepwise. Two peaks containing S-IgA, as revealed by single radial immunodiffusion (17), were eluted at pH 6.6 (0.1 M phosphate buffer). These peaks were pooled and concentrated to a 20-ml volume. Gel filtration was performed on an Ultrogel AcA 34 (LKB, Bromma, Sweden) column (6 by 100 cm) using 0.05 borate buffer at pH 8.0, containing 0.5 M NaCl as the elution buffer. The first peak eluted close to the void volume and contained S-IgA. No other immunoglobulins were present, as revealed by double diffusion against anti-human immunoglobulin antisera (Dakopatts, Copenhagen, Denmark).

Human IgG was commercially obtained from Kabi, Stockholm, Sweden.

Human IgM was prepared from serum. Normal human serum (100 ml) was separated on a Sephacryl S-300 superfine (Pharmacia Fine Chemicals, Uppsala, Sweden) column (10

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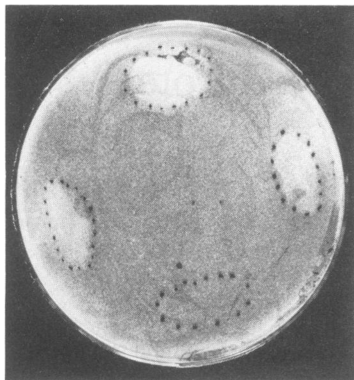

FIG. 1. Bacterial degradation of IgG, made visible by water vapor condensation after removal of the nutrition agar. Decreased wettability, indicating degraded IgG molecules, is seen as whitish spots. The areas with bacterial growth on the nutrition agar are indicated by dotted lines. *S. pneumoniae* EF 2333 was cultivated to the left, and *S. pneumoniae* EF 3447 was cultivated to the right. *S. faecalis* MC4 was used as a positive control (top), and *L. delbrueckii* GH442 was used as a negative control (bottom).

by 100 cm) using phosphate-buffered saline at pH 7.2 as the elution buffer. The protein fraction appearing in the void volume was pooled and dialyzed overnight against 0.1 M phosphate buffer at pH 6.7. Thereafter, ion-exchange chromatography was performed with a DEAE-Sephacel column (2.6 by 50 cm) equilibrated with the same buffer. A pH and ionic strength gradient (0.1 M, pH 6.7; 0.3 M, pH 6.3; volume, 1 liter) was used for elution. A major peak was eluted from the column containing only IgM; no other immunoglobulins were revealed by double diffusion against anti-human immunoglobulin antisera.

Enzyme assay. The principle and the technical performance of the method used for the screening of proteases degrading S-IgA, IgG, and IgM, the thin-layer enzyme assay (TEA) cultivation technique, have been described in detail previously (26, 28). The immunoglobulins were adsorbed from an aqueous solution (0.1 mg/ml) onto the inner surfaces of polystyrene petri dishes (Nunc, Roskilde, Denmark). The surfaces were then rinsed and dried. Blood agar medium (blood agar base no. 2 [Difco Laboratories, Detroit, Mich.], with 5% [vol/vol] defibrinated horse blood added) was applied over the immunoglobulin-coated surfaces. The pneumococci were spotwise inoculated on the agar medium. The enzymes produced by the bacteria during growth reach the protein-coated surface by diffusion. After incubation at 36°C in air containing 10% CO_2, the agar was removed from the dishes, and the immunoglobulin-coated surfaces were rinsed and dried. The wettability of the enzyme-affected part of immunoglobulin-coated polystyrene surfaces was decreased compared with that of the unaffected part (Fig. 1). The difference in wettability was demonstrated by the condensation of water vapor on the surface for 1 min. Each strain was tested at least twice on each class of immunoglobulin included in the study.

Control experiments. The relevance of a decreased wettability as an indicator of degraded immunoglobulins when adsorbed onto a hydrophobic surface was tested in two different ways. Both a reduced ability of anti-immunoglobulin antibodies to bind to the adsorbed immunoglobulins and a reduced thickness of the immunoglobulin protein layer should result from degradation (23, 25, 28).

The ability of anti-immunoglobulin antibodies to bind to enzyme-affected immunoglobulins was tested with an enzyme immunoassay, which was performed essentially as described by Elwing and Nygren (4). The method suggests that peroxidase-labeled antibodies (10 μg/ml in phosphate-buffered saline) are incubated with the adsorbed antigen for 2 h. After rinsing, the surface is immediately coated with agarose (10 g/liter in phosphate-buffered saline at pH 7.0) to which hydrogen peroxide (0.001 g/liter) and phenylenediamine (1 g/liter) have been added. The binding of peroxidase-labeled antibodies to antigen yields a color reaction in the gel.

In this study, degradation of the adsorbed immunoglobulins was indicated by a failure of the color reaction to occur. The peroxidase was coupled to goat anti-human S-IgA, IgG, and IgM (Nordic Immunological Laboratories, Tilburg, The Netherlands). Methods for the preparation and purification of peroxidase-labeled antibodies have been described previously (20).

Thickness measurements of enzyme-affected and unaffected immunoglobulin layers were performed either by using an Isoscope ellipsometer (Isoscope 125, Sagax Instrument AB, Sundyberg, Sweden) as described by Stenberg and Nygren (24) or by a method based on the phenomenon that the interference color of monoxide-treated silicon slides (Sagax slides; Sagax) is dependent on the layer thickness when exposed to natural light (T. Sandström, Ph.D. thesis, technical report no. 128, Chalmers University of Technology, Göteborg, Sweden, 1982). Slides with a reddish-purple color were used for the experiments. Adsorption of immunoglobulins onto the slides made them become bluish, and degradation of the immunoglobulin layers was indicated by a change of this bluish color back to a reddish-purple appearance.

Six strains of *S. pneumoniae* revealing different immunoglobulin-degrading abilities with TEA were used in the control experiments. In addition, *Streptococcus faecalis* MC4 (5), previously shown to degrade IgG (26), and *Lactobacillus delbrueckii* GH442 (our own isolate) were used. The immunoglobulin-coated surfaces also were exposed to trypsin (bovine; E. Merck AG, Darmstadt, Germany) as described for the TEA drop technique (28).

The control experiments showed that when a decreased wettability was registered with TEA, a degradation of the immunoglobulins could be verified with the enzyme immunoassay and by the thickness measurements in all cases.

Statistical method. Statistical significance was calculated by the chi-square test of probability (1).

RESULTS

Of 102 strains of *S. pneumoniae* (Table 1), the ability to degrade S-IgA was revealed in 50 strains; the ability to degrade IgG was revealed in 84 strains, and the ability to degrade IgM was revealed in 96 strains (Table 2). A concurrent degradation of S-IgA, IgG, and IgM was revealed in 38 strains. A concurrent degradation of IgG and IgM but not S-IgA was revealed in 44 strains. A concurrent degradation of S-IgA and IgG but not IgM was revealed in one strain, and a concurrent degradation of S-IgA and IgM but not IgG was revealed in nine strains. Three strains degraded only S-IgA, one strain degraded only IgG, and five strains degraded only IgM. One strain was unable to degrade any of the immunoglobulins.

The proteolytic activity of the *S. pneumoniae* strains caused a smaller decrease of the wettability of the immuno-

TABLE 1. Strains of *S. pneumoniae* used in the study

Strain origin	Serotype	No. of strains
Acute meningitis	4	1
	6	1
	6B	1
	9	1
	14	4
	18C	1
	19F	4
	23F	2
	25	3
Acute pneumonia (strains from blood)	3	10
	5	2
	9V	1
	9N	1
	14	6
	19A	1
	20	1
	25	6
Acute pneumonia (strains from bronchial secretion)	3	2
	6A	2
	13	2
	18	1
	23	1
	Rough	1
Acute otitis	3	2
	4	1
	6A	5
	6B	4
	9V	1
	11A	2
	14	1
	19	1
	19F	11
	22F	1
	23	1
	23F	6
	Rough	1
Symptomless carriers	6A	1
	6B	1
	14	2
	15B	3
	18C	2
	23F	1

globulin-coated surfaces than *S. faecalis* MC4 or the trypsin solution. This difference was most evident on the S-IgA-coated surfaces.

The incidence of immunoglobulin degradation by the *S. pneumoniae* strains as related to their origin is given in Table 2. Strains isolated from patients with acute pneumonia showed different abilities to degrade S-IgA. Thus, more strains originating from bronchial secretion degraded S-IgA than did strains originating from blood ($P < 0.01$). S-IgA-degrading ability was also more frequently observed in strains from symptomless carriers than in strains from patients with acute otitis or from the blood of patients with acute pneumonia ($P < 0.05$ and $P < 0.01$, respectively). The ability to degrade IgG and IgM was not observed more often in strains isolated from infected carriers than from symptomless carriers. Neither were any differences of the IgG- and IgM-degrading ability found in *S. pneumoniae* strains isolated from patients with various types of infections.

TABLE 2. Degradation of human S-IgA, IgG, and IgM by *S. pneumoniae* strains of different origins

Strain origin	No. of strains tested	No. (%) of positive strains		
		S-IgA	IgG	IgM
Acute meniningitis	18	12 (67)	9 (50)	15 (83)
Acute pneumonia (strains from blood)	28	9[a] (32)	25 (89)	15 (89)
Acute pneumonia (strains from broncheal secretions)	9	8[b] (89)	9 (100)	9 (100)
Acute otitis	37	13[c] (35)	34 (93)	37 (100)
Symptomless carriers	10	8[d] (80)	7 (70)	10 (100)
Total	102	50 (49)	84 (82)	96 (94)

[a] Level of significance: *a/b*, $P < 0.01$; *a/d*, $P < 0.01$; *c/d*, $P < 0.05$.

Table 3 shows the immunoglobulin-degrading ability of *S. pneumoniae* with nine or more strains of the same serotype. No correlation was found between serotype affiliation and immunoglobulin-degrading ability.

DISCUSSION

In the present study, production of enzymes degrading S-IgA, IgG, and IgM was demonstrated in *S. pneumoniae*. The 102 strains utilized were isolated from patients with acute meningitis, pneumonia, and otitis media, as well as from symptomless carriers, and were of various serotypes.

Previous studies on microbial immunoglobulin-degrading enzymes have usually been carried out with immunoelectrophoresis, making it possible to identify the degradation products (9, 11, 14, 16, 22). Although specific, the immunoelectrophoresis technique is considered to be insensitive and rather laborious when a great number of bacterial strains are to be tested.

The present study was performed with a plate assay, the TEA cultivation technique, which in previous studies has been found to be both sensitive and convenient for the screening of microbial proteases (26, 27). The assay uses proteins adsorbed to a polystyrene surface as the enzyme substrate, and degraded protein is demonstrated by water vapor condensation. The wettability of the enzyme-affected part of the protein-coated surface is decreased compared with that of the unaffected part. The relevance of wettability as an indicator of immunoglobulin degradation was verified

TABLE 3. Degradation of human S-IgA, IgG, and IgM by proteases from *S. pneumoniae* of seven common serotypes

Serotype	No. of strains tested	No. (%) of positive strains		
		S-IgA	IgG	IgM
3	14	4 (29)	14 (100)	14 (100)
6A	8	5 (63)	8 (100)	8 (100)
6B	6	3 (50)	5 (83)	6 (100)
14	13	5 (38)	8 (62)	11 (85)
19F	15	8 (53)	13 (87)	14 (93)
23F	9	5 (56)	6 (67)	9 (100)
25	9	3 (33)	9 (100)	9 (100)

by the use of peroxidase-labeled antibodies against the different immunoglobulins (4) and by thickness measurements of the immunoglobulin layers (24). Thus, decreased wettability of the immunoglobulin-coated polystyrene surfaces was accompanied by an inability of anti-immunoglobulin antibodies to bind to the immunoglobulins and a decreased thickness of the immunoglobulin layers. The TEA cultivation technique was therefore judged to be suitable for the screening of immunoglobulin-degrading proteases.

With the use of the immunoelectrophoresis technique, *S. pneumoniae* has been shown by Male (16), Kilian et al. (11), and Mulks et al. (18) to cleave human IgA1 myeloma proteins and to cause a partial cleavage of human S-IgA. These authors observed no degradation of IgG or IgM. In the present study, however, IgG and IgM degradation was detected. This discrepancy in results may be due to different assay conditions and different sensitivities in the assays used. It may also be due to the fact that only a few strains were tested for IgG- and IgM-degrading enzymes in the previous studies. Meanwhile, the results obtained in this work indicate that IgG- and, in particular, IgM-degrading ability is a characteristic of most strains of *S. pneumoniae* (Table 2).

The partial cleavage of S-IgA by *S. pneumoniae* (11, 16, 18) was found by Kilian et al. (10) to be dependent on a resistance of the IgA2 molecules to being cleaved by IgA1 protease and on the occurrence of protease-neutralizing antibodies. The IgA2 molecules usually constitute a considerable portion of S-IgA (3). A mixture of susceptible and nonsusceptible IgA molecules and of protease-neutralizing antibodies in S-IgA may also explain our observations that *S. pneumoniae* changed the wettability of S-IgA-coated surfaces to a lesser extent than did IgG- and IgM-coated surfaces. Certainly the degradation of S-IgA by *S. pneumoniae* was more easily detected by the use of peroxidase-labeled S-IgA antibodies, but their use in large-scale screening is hampered by their high cost.

The endoglucosidases (neuraminidase, β-galactosidase, exoglucosaminidase, and endoglucosaminidase) produced by *S. pneumoniae* (12) have been shown to remove carbohydrates from IgA1 proteins (10) and can be expected to remove carbohydrates from other immunoglobulins. Removal of the carbohydrates from the immunoglobulins would also reduce the wettability and might give rise to false-positive results with TEA. However, the decreased wettability was accompanied by an inability of anti-immunoglobulin antibodies to bind. Since such antibodies are mainly, if not entirely, antiprotein, the above results show that *S. pneumoniae* degraded the immunoglobulins when a decreased wettability was obtained with TEA.

Concurrent degradation of S-IgA, IgG, and IgM was revealed with the TEA in 38 of 102 strains. Degradation of two of the immunoglobulins was revealed in 54 strains, and degradation of one of the immunoglobulins was revealed in 9 strains. Thus, 90% of the strains tested had the ability to degrade at least two of the immunoglobulins. Furthermore, the data indicate that the degradation of S-IgA, IgG, and IgM by *S. pneumoniae* strains is most probably due to proteases with different specificities.

In accordance with pneumococcal IgA1-degrading ability (11, 16, 18), there was no correlation between the IgG- and IgM-degrading ability of the strains tested and their origin or serotype affiliation. However, S-IgA-degrading ability was observed more frequently in the strains isolated from symptomless carriers and bronchial secretions of patients with acute pneumonia than in the strains isolated from patients with acute meningitis or acute otitis media or from the blood of patients with acute pneumonia. Therefore, it seems rational to attach significant biological value to the S-IgA proteases for the bacterial colonization of mucosal surfaces.

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